# US Environmental Protection Agency
# Office of Pesticide Programs

**Office of Pesticide Programs**
**Microbiology Laboratory**
**Environmental Science Center, Ft. Meade, MD**

**Standard Operating Procedure for**
**Method for the Evaluation of Antimicrobial Activity of Hard, Non-porous Copper-Containing Surface Products**

**SOP Number: MB-41-00**

**Date Revised: 09-26-22**

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| Title | Method for the Evaluation of Antimicrobial Activity of Hard, Non-porous Copper-Containing Surface Products |
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| SOP Number | MB-41-00 |
| Title | Method for the Evaluation of Antimicrobial Activity of Hard, Non-porous Copper-Containing Surface Products |
| Scope | Describes the methodology used for evaluating the durability and efficacy of copper-containing surfaces against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and viruses. |
| Application | This methodology described in this SOP is limited to copper-containing surfaces against the prescribed test microbes. |

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**Method for the Evaluation of Antimicrobial Activity of Hard, Non-porous Copper-Containing Surface Products**

## Scope

The Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recommends that applicants utilize this test method to support efficacy requirements for the registration of hard, non-porous copper-containing surface products with non-food contact surface antimicrobial claims and are designed to be supplements to standard disinfection practices. The claim is intended for indoor use only including claims for "continuous reduction of bacteria and viruses." The test method applies to solid copper products, impregnated copper products, and copper coated products (sold as coated pre-market). The test method provides guidance for the evaluation of durability and efficacy of the copper surfaces against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and viruses; the test method can be adapted for additional organisms. A minimum 3 log reduction of test organisms within 1-2 hours is the required level of performance. This test method is a revised version of the interim method posted on 10/26/2020 (EPA-HQ-OPP-2020-0529).

## Method Overview

In brief, the test method is comprised of two parts: 1) abrasion and chemical treatment, and 2) product efficacy. The method specifies the use of 1" × 1" copper (product) carriers and stainless steel control carriers. Carriers are exposed to abrasion and chemical treatment five times a day, five days a week for six consecutive weeks. The abrasion and chemical exposure processes are intended to represent a degree of normal and relevant physical wear, as well as reproduce potential effects resulting from repeated exposure of copper-containing surfaces to three different biocidal materials (chemical solutions). The impact of the abrasion and chemical exposure on the integrity of product carriers is documented – these carriers are considered "exposed" carriers. Following completion of abrasion and chemical exposure, each product and control carrier receives a 20 µL mixture of the test organism and soil load. Following a 1-2 hour contact time, the carriers are neutralized, and the number of viable microorganisms is determined quantitatively. The log reduction (LR) in the viable test organisms on exposed carriers is calculated in relation to the viable test organisms on the unexposed control carriers. The impact of the abrasion and chemical exposure on product efficacy is also determined by comparing carriers not exposed to abrasion and chemical treatment.

Appropriate safety procedures should always be used when working with laboratory test systems which include human pathogenic microorganisms. Laboratory safety is discussed in the current edition of "Biosafety in Microbiological and Biomedical Laboratories (BMBL)" from the subject matters experts within the U.S. Department of Health and Human Services (HHS), including experts from the Centers for Disease Control and Prevention (CDC) and National Institutes for Health (NIH).

**Table of Contents**

1) Terminology……... 4
2) Apparatus…….. 4
3) Bacterial Reagents……...…….. 5
4) Viral Reagents…… 6
5) Common Reagents……. 7
6) Carriers……..... 8
7) Chemical Exposure and Abrasion Treatment Process……... 9
8) Preparation of Test Cultures: *P. aeruginosa and S. aureus*……... 12
9) Preparation of Test Culture: Viruses……. 13
10) Efficacy Assessment…… 13
11) Bacterial Recovery……... 15
12) Virus Recovery…… 16
13) Calculations/Data Analysis……..... 17
14) References…… 19
Appendix A: Preparation of Bacterial Frozen Stock Cultures……..……. 20
Appendix B: Examples of Failed Physically Screened Carriers …….……..……..... 22
Appendix C: Example of Dry Inoculated Stainless Steel Carrier ……. 23
Appendix D: Bacterial Neutralization Assay ….……... 24
Appendix E: Cytotoxicity Determination ……...……...……..... 26
Appendix F: Viral Neutralization Assay …….……. 28
Appendix G: Synthetic Broth Recipe……. 30

## 1. Terminology

a. *Control carriers, n*—stainless steel carriers

b. *Cycle (abrasion cycle), n*—a series of 12 sequential, single passes across the surface of carriers.

c. *Exposed carriers, n*—carriers that are exposed to chemical exposure and abrasion cycles via a non-scratch scour pad on the surface of the abrasion tester tray.

d. *Min, n*—minutes.

e. *Pass, n*—within an abrasion cycle, one movement across the surface of the carriers.

f. *S, n*—seconds.

g. *Product carriers, n*—copper or copper coated carriers

h. *Unexposed carriers, n*—carriers that are not exposed to chemical exposure and abrasion cycles via a non-scratch scour pad on the surface of the abrasion tester tray.

## 2. Apparatus

a. *-20°C Freezer.* Used for storage of soil aliquots.

b. *-80°C Freezer.* Used for storage of frozen stock cultures.

c. *0.2 µm pore diameter polyethersulfone (PES) membrane filters, 47 mm diameter*. Used for recovery of test microbe (bacteria). Filtration units (reusable or disposable) may be used.

d. *3M Scotch Brite Non-scratch scour pads.* UPC 0 5114137319 5

e. *Abrasion Boat*. Used to abrade carriers with non-scratch scour pad attached.

f. *Carriers, Copper (product) carriers*. Die/machine cut 1” × 1” square from copper test product, single use.

g. *Carriers, Stainless steel (control) carriers.* Die/machine cut 1” × 1” square made from sheet stainless steel (AISI #304). The carriers should physically match the product carriers as closely as possible with respect to thickness, degree of polish and/or brushed surface machining, etc. Carriers are single use.

h. *Centrifuge* (with rotor capable of achieving 5,000 g). Used for test culture preparation.

i. *Certified timer*. Readable in minutes and seconds, for tracking of timed events and intervals.

j. *Conical tubes* (e.g., 15 mL, 50 mL). Capable of being centrifuged at 5,000 g. Used for culture preparation and neutralization.

k. *Cryovials*. Used for storage of frozen stock culture.

l. *Dilution tubes* (glass/plastic tubes). Used for preparing serial dilutions.

m. *Environmental chamber*. Used for efficacy testing, to hold carriers during microbe contact time at 21±3°C and 30-40% relative humidity.

n. *Filter paper* (Whatman No. 2). Used to line Petri dishes associated with carriers.

o. *Forceps, sterile.* Used to handle carriers and membrane filters.

p. *Gardco Model D10V or comparable abrasion instrument*. Used to simulate wear on carriers.

q. *Identification system (optional)*. Used for appropriate identification of test microbes (e.g., VITEK identification system).

r. *Incubator.* Used to incubate test cultures and growth medium plates at 36±1°C.

s. *Incubator with 5% $CO_2$*. Used for growing cell lines and viruses (when applicable).

t. *Liquid nitrogen dewar or comparable freezer capable of long-term storage of cell lines.* Used for storing viral cell lines.

u. *Kimwipes*. *Lint free cloth*; used for drying and cleaning carriers.

v. *Microcentrifuge tubes*. Used for storage of soil single use aliquots.

w. *Microscope* (e.g., 100x optics and 10x ocular). Used for microbial observation.

x. *Micropipettes, calibrated*. Used with corresponding tips for preparing dilutions.

y. *Petri dishes.* Glass/plastic used as a flat surface for inoculating and incubating carriers. Also used with filter paper for carrier drying, storage, and chemical treatment.

z. *Positive displacement pipette, 20 µL calibrated.* Used with corresponding tips for carrier inoculation.

aa. *Refrigerator* (2-8°C). Used to store media and post-incubated plates.

bb. *Serological Pipettes.* Used for removing/adding larger volumes of liquid (e.g., 10 mL, 25 mL).

cc. *Sonicator* (capable of producing 45 Hz). Used for removal of organism from carriers. If necessary, verify the sonicator to determine the impact of sonication on the culture by placing the standardized broth culture into sonicator for 5 min, serially dilute, and recover. Compare sonicated counts to a non-sonicated control. The sonicated and non-sonicated counts should be comparable.

dd. *Spray Bottle*. Used to apply chemical treatments (solutions A, B, and C) to carriers.

ee. *Titration kit (e.g., Hach digital titrator).* Used for measuring total chlorine.

ff. *Vacuum source* (in-house line or suitable vacuum pump). Used to facilitate rapid membrane filtration.

gg. *Vortex*. Used for vortex mixing of various solutions including carriers.

**3. Bacterial Reagents**

a. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus* (ATCC #6538)

b. Culture media for *P. aeruginosa* and *S. aureus.*

i. *10% (w/v) dextrose solution.* Used as a supplement to synthetic broth. Add 5.0 g dextrose to 50 mL de-ionized water (to bring to volume in a volumetric flask) and mix by stirring. Filter sterilize the solution using a 0.2 µm filter. Store the sterile solution at 2-8°C for up to 30 days.

ii. *Phosphate buffered saline stock solution (e.g., 10X).* Used for preparing 1X phosphate buffered saline. The stock solution has a pH of approximately 7.2±0.2.

iii. *Phosphate buffered saline (PBS), 1X.* Used for dilution blanks and filtration. PBS has a pH of approximately 7.0±0.5.

iv. *Selective media (optional)*. Cetrimide agar (*P. aeruginosa*) and Mannitol salt agar (*S. aureus*). See Table 5 in Appendix A for use. Purchase from a reputable source or prepare according to manufacturer's instructions.

v. *Synthetic broth (SB).* Used as the growth medium for test cultures. Commercial media (HIMEDIA, Synthetic Broth, AOAC, #M334-500G). Store prepared SB at 2-8°C.

1. Alternatively, SB made in-house per the recipe provided in Appendix G and AOAC Methods 955.15, 964.02, and 955.14 may be substituted.

vi. *Trypticase Soy Agar (TSA)*. Used as a recovery medium for bacterial enumeration and purity checks. Prepare TSA according to manufacturer's instructions.

1. Equivalent commercially prepared agar culture medium may be purchased.

vii. *Trypticase Soy Agar with 5% sheep blood (BAP).* Used for performing streak isolation of microbial cultures as a purity check (quality control purposes).

viii. *Trypticase Soy Broth (TSB), (30g/L).* Used for rehydrating lyophilized/frozen vegetative culture of test microorganism. Prepare TSB according to manufacturer's instructions.

ix. *TSB with 15% (v/v) glycerol.* Used as a cryoprotectant solution. Suspend 7.5 g TSB in 212.5 mL de-ionized water. Using a positive displacement pipette, dispense 37.5 mL glycerol and stir, warm slightly to dissolve. Dispense into bottles and steam sterilize for 15 min at 121°C.

1. Alternatively, purchase broth from a reputable source or prepare according to manufacturer's instructions.

c. *Gram stain kit.* Used for diagnostic staining.

**4. Viral reagents**

a. Test virus: use appropriate virus to be claimed on the label

b. Cell line: use an appropriate cell line for virus selected for efficacy testing.

c. Viral media

i. *Complete Growth Media (CGM).* Consisting of Minimum Essential Media and FBS or other medium specified for the test virus. Used for cell line propagation,

viral propagation, and serial dilution. Antibiotics and/or antifungals may be added to reduce potential contamination.

1. *Minimum Essential Media (MEM).* Liquid or powder form (e.g., Eagle's or Dulbecco's). Used to prepare complete growth media. Prepare per manufacturer's guidelines.
2. *Heat Inactivated Fetal Bovine Serum (FBS)*, compatible for use with cell lines. Often used to prepare CGM.

ii. *Dulbecco's Phosphate buffered saline (DPBS)*, or other equivalent buffer (e.g., PBS, Earle's Balanced Salt Solution). Prepare per manufacturer's guidelines.

d. *Antibiotic/antifungal.* 100x Amphotericin B/Penicillin/Streptomycin solution or other equivalent antibiotic/antimycotic solution. May be used to prevent contamination of cell culture.

**5. Common Reagents**

a. *95-98% ethanol*. Used to decontaminate carriers prior to efficacy testing.

b. *De-ionized (DI) water.* Used for preparation of reagents, media, and rinsing test solutions off carriers.

c. *Liquinox or equivalent non-ionic solution.* To clean carriers.

d. *Neutralizer.* Various confirmed neutralizers may be used, including letheen broth. If necessary, other ingredients may be added to letheen broth. Purchase letheen broth from a reputable source or prepare according to manufacturer's instructions.

i. The recommended neutralizer for the viral test system is the same medium used to grow the virus (e.g., CGM). If the neutralization confirmation assay demonstrates that CGM is ineffective, other neutralizers may be used.

e. *Soil Load, 3-part.* The standard soil load to be incorporated in the final test suspension.

i. *Bovine Serum Albumin (BSA).* Add 0.5 g BSA (radio immunoassay (RIA) grade or equivalent, CAS# 9048-46-8) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter polyethersulfone membrane filter, aliquot, and store frozen at -20±2°C for up to one year. Aliquots are single use only; do not refreeze once thawed.

ii. *Yeast Extract.* Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter polyethersulfone membrane filter, aliquot, and store frozen at -20±2°C for up to one year. Aliquots are single use only; do not refreeze once thawed.

iii. *Mucin.* Add 0.04 g mucin (from bovine submaxillary gland, CAS# 84195-52-8) to 10 mL of PBS, stir or vortex-mix until thoroughly dissolved, and pass through a 0.2 µm pore diameter polyethersulfone membrane filter, aliquot, and store frozen at -20±2 °C for up to one year. Note: mucin may require vigorous stirring or vortex-mixing to fully dissolve. Aliquots are single use only; do not refreeze once thawed.

iv. See section 8j and 9e for addition of soil load to bacterial and viral inoculum respectively.

f. Treatments used in chemical exposure of carriers

i. *Treatment A.* A 3000±150 ppm sodium hypochlorite (NaOCl) solution (e.g., Sigma-Aldrich reagent grade sodium hypochlorite) prepared in deionized water. Verify the final concentration of the treatment using a suitable titration method (e.g., Hach digital titrator). Identify concentration in the study report.

ii. *Treatment B*. Use an EPA-registered hospital disinfectant product containing hydrogen peroxide (between 3.0% and 6.0%) and peracetic acid as active ingredients that allows spray application to hard, non-porous surfaces. The treatment concentration for the peracetic acid component is not limited to a defined range. Identify product in the study report.

iii. *Treatment C*. Use an EPA-registered hospital disinfectant product with quaternary ammonium compound as the active ingredient labeled as a one-step cleaner/disinfectant that allows spray application to hard, non-porous surfaces. Identify product in the study report.

**6. Carriers**

a. The following section provides guidelines for preparation of both stainless steel control carriers and product carriers. Two production lots of the product should be used to evaluate efficacy. Lot 1 is used for both abrasion/chemical treatments and efficacy. Lot 2 is used for efficacy testing only. See Table 1 for a summary of carrier distribution. "Exposed" refers to carriers subjected to the physical abrasion/chemical treatment, while "unexposed" refers to those carriers not subjected to the physical abrasion/chemical treatment.

**Table 1. Carrier Distribution for Testing Copper-Containing Surface Products**

| | **Carrier Type** | **# of carriers for *S. aureus*** | **# of carriers for *P. aeruginosa*** |
|---|---|---|---|
| **Lot 1** | Exposed Product | 5 per exposure*<br>(15 total) | 5 per exposure*<br>(15 total) |
| | Exposed Stainless Steel | 3 per exposure*<br>(9 total) | 3 per exposure*<br>(9 total) |
| | Unexposed Product | 3 | 3 |
| | Unexposed Stainless Steel | 3 | 3 |
| **Lot 2** | Unexposed Product | 5 | 5 |
| | Unexposed Stainless Steel | 3 | 3 |

*3 separate treatments (A, B, and C) with abrasion

b. Screen and clean carriers prior to abrasion/chemical exposure and maintain production lot identity throughout the testing process.

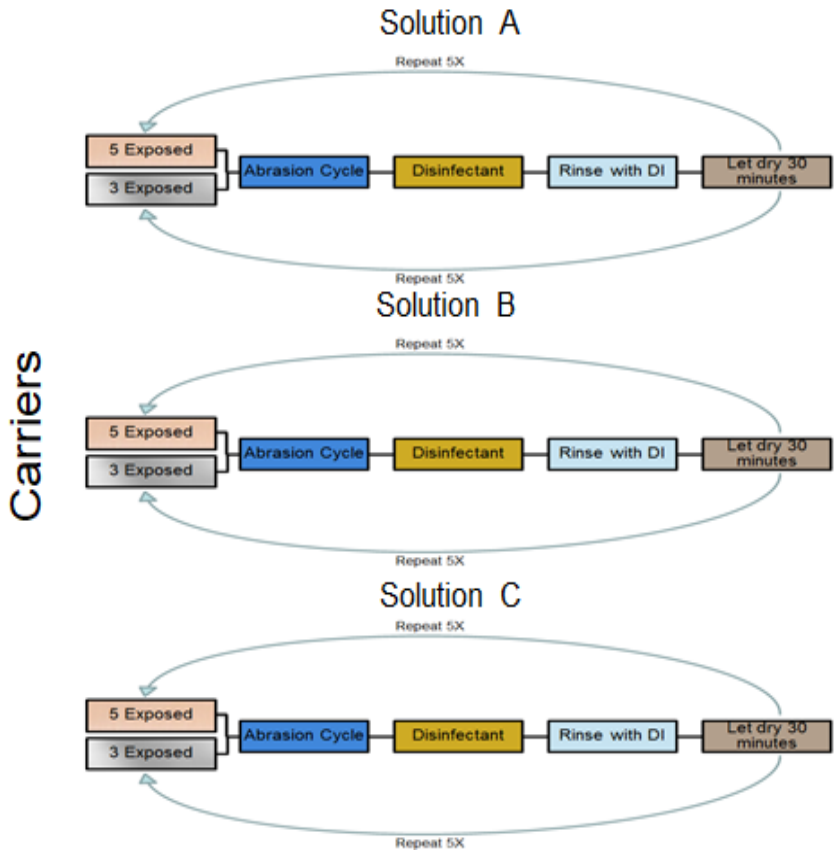
c. Inspect each carrier to ensure uniformity. Discard carriers with visible surface or edge abnormalities (e.g., corrosion/rust, chipping, gouges, or deep striations, etc.); refer to examples in Appendix B.

d. Soak screened carriers in a non-ionic detergent solution (e.g., Liquinox) for 2-4 hours to degrease and then rinse thoroughly in deionized water. Gently wipe with a clean lint-free cloth and allow to completely dry.

e. Prepare at least one additional product carrier and one additional stainless steel control carrier for sterility assessment.

## 7. Chemical Exposure and Abrasion Treatment Process

**Figure 1.** Abrasion and Chemical Exposure Treatment Diagram – for exposed carriers of a single production lot for 1 day




a. Preparation for physical abrasion.

i. Select and prepare carriers as described in Section 6 of this document prior to the abrasion and chemical exposure process.

ii. Orient individual carriers with the exposed copper surface side-up (i.e., the bactericidal copper-containing surface). Maintain this orientation throughout the exposure treatment. For the stainless steel control carriers, either side of the carrier may be selected but maintain the orientation throughout the exposure treatment.

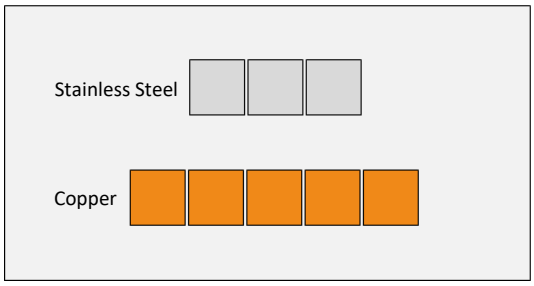
iii. A single abrasion and chemical exposure treatment is twelve passes of an abrasive material (i.e., a non-scratch scour pad) against the product surface followed by a 20-minute exposure of the product surface to a specific treatment.

iv. Perform exposure treatments on Lot 1 five times per day and five days a week for six consecutive weeks, resulting in a total of 150 abrasion and chemical exposures.

v. Maintain all product and stainless steel control carriers under comparable conditions during each abrasion and chemical treatment exposure treatment. Perform the exposure treatment of the carriers at room temperature (21±3°C).

b. Conducting the Abrasion Treatment

**Figure 2.** Recommended Abrasion Process – Carrier Configuration




i. Perform the abrasion exposure with the Gardco, Model D10V abrasion tester or comparable equipment. Consult the owner's manual to ensure proper set up and maintenance and calibration.

ii. Calibrate instrument to achieve 2-2.5 seconds for one pass and 24-30 seconds per abrasion cycle.

iii. Attach the pad to the abrasion tester as specified in the manual or using double sided tape. Each exposed treatment must have its own abrasion pad. Do not use the same abrasion pad for product and stainless steel control carriers. Replace abrasion pads daily.

iv. The total weight of the abrasion boat plus the abrasion material must be 415 to 435 g.

v. Load carriers onto abrasion instrument and conduct abrasion cycle.

vi. Product and stainless steel control carriers should be situated in parallel with one another (see Figure 2) or abraded separately.

vii. Do not adhere carriers to the abrasion instrument; use a template with cut-outs or similar device to hold the carriers in place during abrasion process.

viii. One abrasion cycle consists of twelve passes of the abrasive pad against the carrier surface (the pad to contact the carrier surface twelve times).

ix. Following the abrasion cycle, wipe the carriers with a clean, dry cloth (Kimwipe), and subject the carriers to the chemical treatment.

c. Chemical Treatment

**Table 2.** Carrier Exposure to Chemical Treatments

| **Treatment A** | **Treatment B** | **Treatment C** |
|---|---|---|
| Sodium Hypochlorite (NaOCl) | Hydrogen peroxide | Quaternary ammonium |
| 5 exposed product carriers | 5 exposed product carriers | 5 exposed product carriers |
| 3 exposed stainless steel carriers | 3 exposed stainless steel carriers | 3 exposed stainless steel carriers |

i. Place the carriers, product and stainless steel control carriers, exposed surface up on a flat surface (e.g., inside a Petri dish) with absorbent material (e.g., filter paper) to absorb excess chemical treatment. Apply each chemical treatment to the exposed carriers by spraying two to three pumps of the appropriate treatment at 6-8 inches from the carrier surface.

ii. Allow each carrier to be in contact with the chemical treatment for 20±1 min at room temperature.

iii. After the contact period, rinse each carrier thoroughly with DI water, air dry, and store uncovered at room temperature until the next exposure cycle.

1. Wait a minimum of 30 min between each exposure cycle.

d. Following the 150 abrasion/chemical exposures, rinse all carriers thoroughly with DI water, air dry, and store at room temperature in individual covered Petri dishes lined with filter paper. Visually inspect carrier surfaces (for the designated production lot). Describe and document any physical disruption or degradation of the exposed product surface (e.g., decolorization, cracking, peeling, and chipping).

e. After completing the abrasion/chemical exposure (for Lot 1 only) immerse all product and stainless steel control carriers in 95-98% ethanol for approximately 10 min. Using sterile forceps, remove individual carriers and place face up in pre-sterilized Petri dishes (one carrier per dish) lined with filter paper. Allow carriers to dry with lid open. Orient individual carriers with the treated exposed side (i.e., copper surface) up; maintain this orientation. Handle carriers aseptically.

f. Include all carrier storage conditions (temperature and humidity range) in the study report.

g. Initiate efficacy testing within 7 days of completion of the final abrasion/chemical exposure process.

**8. Preparation of Test Cultures: *P. aeruginosa* and *S. aureus***

a. Refer to Appendix A for preparation of the frozen stock cultures.

b. Defrost a cryovial rapidly to avoid loss in the viability of the preserved cells. Each cryovial is single use only.

c. No more than 15 min prior to inoculation, use a calibrated micropipette to aseptically add 0.1 mL of 10% sterile dextrose (w/v) solution to each 10 mL tube of SB in a 20×150 mm glass culture tube with a Morton closure; mix well.

d. Using a calibrated micropipette, add 100 µL of defrosted stock culture to the same tube containing 10 mL SB + 0.1 mL dextrose, briefly vortex-mix and incubate for 24±2 h at 36±1°C.

   i. In addition, inoculate TSA or TSA with 5% sheep blood plate from the inoculated tube and streak for isolation. Incubate plate with the test culture.

   ii. Incubate without disrupting the culture (i.e., do not move culture while incubating).

e. Following incubation, use the SB cultures to prepare a test suspension for each organism. Record results from the streak isolation plate.

f. The 24±2 h culture should exhibit a titer of at least $10^8$ CFU/mL. Record time of culture harvest.

   i. For *P. aeruginosa*, inspect culture prior to harvest; visible pellicle on the surface of the culture is expected to form during incubation (record its presence). Discard the culture if pellicle has been disrupted (fragments in culture).

      1. Remove visible pellicle on surface of medium and around associated interior edges of the tube with vacuum suction.

      2. Using a serological pipette, withdraw the remaining broth culture (approx. 7-8 mL) avoiding any sediment on the bottom of the tube and transfer it into a 15 mL conical centrifuge tube.

      3. Record approximate volume harvested and transferred to 15 mL conical tube.

   ii. For *S. aureus*, briefly vortex the 24±2 h culture and transfer to a 15 mL centrifuge tube.

      1. Record approximate volume harvested and transfer to 15 mL conical tube

g. Within 15 minutes, centrifuge the 24±2 h harvested broth cultures at 5,000×g for 20 min. Record time of centrifugation.

h. Remove the supernatant without disrupting the pellet. Once supernatant is removed, re-suspend the pellet in 5-10 mL PBS. Record resuspension volume and time of resuspension.

i. If necessary, disrupt the pellet using vortex-mixing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely. If necessary, add 1 mL of PBS to the pellet first to aid in disaggregation.

i. For efficacy testing, further dilute the 5-10 mL of resuspended culture in PBS as necessary to achieve a mean control carrier count level of 4.0-5.0 logs CFU/carrier for *P. aeruginosa* and *S. aureus*. Each inoculated carrier must be within this range following the 2-hour exposure time for a valid test. Record time of test suspension dilution.

i. Optical density/absorbance (e.g., 650 nm) may be used as a tool to monitor/adjust the diluted test suspension; record if measured.

j. Use the diluted culture within 30 min to prepare the final test suspension with the addition of the soil load.

i. Vortex-mix the test suspension for 10-30 s.

ii. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette:

1. 25 µL BSA stock
2. 35 µL yeast extract stock
3. 100 µL mucin stock
4. Vortex soil suspension for 10 s prior to adding microbial test suspension.
5. 340 µL microbial test suspension.

k. Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 30 min of preparation. Record time of final test suspension preparation.

i. Within the 35 carriers, there are a total of 9 independent "sets" of carriers (5 sets of 3 carriers, 4 sets of 5 carriers). A 90-minute use period for the culture with the 3-part soil load provides the opportunity to stagger inoculations, 10 min per carrier set.

ii. Inoculate 1 carrier from Control Set #1 first. Continue inoculations with Treated Set #1, Treated Set #2, Control Set #2; then inoculate the remaining 2 carriers from Control Set #2.

l. Streak inoculate an agar plate (TSA or TSA with 5% sheep blood) with a loopful of the final test suspension. Incubate at 36±1°C for 48±4 h and visually examine for purity.

**9. Preparation of the Test Culture: Viruses**

a. Propagate the test virus on the appropriate cell line.

i. Note: Concentration of the test virus stock (~100,000×g for 4 h at 4ºC) may be necessary to achieve adequate control counts.

b. Defrost a cryovial rapidly to avoid loss in the viability of the preserved virus (e.g., place in a 37ºC water bath and use within 15 min after thawing). Record time placed in water bath.

c. Dilute the virus stock with CGM to achieve control counts in the range of 4.0 to 5.0 logs virus particles/carrier. Record time of virus stock dilution.

d. Use the diluted virus within 30 min to prepare the final test suspension with the addition of the soil load.

e. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette (smaller volumes may be used proportionally):

   i. 25 µL BSA stock

   ii. 35 µL yeast extract stock

   iii. 100 µL mucin stock

   iv. Vortex soil suspension for 10 s prior to adding viral test suspension.

   v. 340 µL virus test suspension

f. Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 30 min of preparation. Record time of final test suspension preparation.

   i. Within the 35 carriers, there are a total of 9 independent "sets" of carriers (5 sets of 3 carriers, 4 sets of 5 carriers). A 90-minute use period for the culture with the 3-part soil load provides the opportunity to stagger inoculations, 10 min per carrier set.

   ii. Inoculate 1 carrier from Control Set #1 first. Continue inoculations with Treated Set #1, Treated Set #2, Control Set #2; then inoculate the remaining 2 carriers from Control Set #2.

**10. Efficacy Assessment**

a. Conduct efficacy testing on all product and stainless steel control carriers within 7 days of completing the abrasion/chemical exposure cycles.

b. Perform the neutralization assay for all organisms prior to testing to demonstrate the neutralizer's ability to inactivate the copper and copper ions; see Appendix D (bacteria) or Appendix F (viruses).

c. In preparation for efficacy testing, it is advisable to determine the appropriate dilution of the test suspension that will ensure control counts in the appropriate range after drying by inoculating three stainless steel control carriers, placing them in the environmental chamber for 1-2 hours, and determining the counts per carrier.

d. Prepare test cultures per Section 8 (bacteria) or Section 9 (viruses) to achieve a final target control count on stainless steel control carriers of 4.0-5.0 logs CFU or viral particles per carrier after the 1-2 hour contact time.

e. Set environmental chamber to achieve 21±2°C and 30-40% relative humidity during the 1-2 h contact period; record temperature and humidity over the contact period.

f. Record the time for all timed events.

g. Process product carriers first and stainless steel control carriers last.

h. Inoculate each carrier with 20 µL of final test culture using a calibrated pipette. Spread the inoculum to within 1/8 inch of the edge of each carrier using a sterile transfer loop or the pipette tip. Place in environmental chamber within 10 minutes of inoculation.

i. Allow carriers to remain in a flat, horizontal position with the lid on the Petri dish in the environmental chamber for 1-2 hours. Refer to Appendix C for picture of dried inoculum on carrier.

**11. Bacterial Recovery**

a. Following the contact time, sequentially and aseptically transfer each carrier to a 50 mL conical tube containing 20 mL of the appropriate neutralizer solution. Remove and neutralize all carriers within 10 minutes

    i. The tube with the neutralizer and the carrier represents the $10^0$ dilution.

b. After all the carriers have been transferred into the neutralizer, vortex-mix for 30 s then sonicate for 5 min±30 s at 45 Hz to suspend any surviving organism in the neutralizer. If necessary, refer to section 2cc for sonicator verification.

c. Initiate serial dilutions of the neutralizer tubes in PBS within 30 min.

d. Initiate filtration within 30 min of preparing dilutions.

e. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS. Apply vacuum to filter contents; leave the vacuum on for the duration of the filtration process regardless of filtration apparatus used (e.g., filter manifold, single filter unit).

f. Use separate PES membrane filters for each eluate; however, the same filtration unit may be used for processing eluates from a given carrier set starting with the most dilute sample first.

g. Pour the eluate into the filter unit. Rinse tubes (conical tube and/or dilution blank) once with ~10 mL PBS, briefly vortex-mix, and pour into filter unit.

h. Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus.

i. Rinse the inside of the surface of the funnel unit with ~20 mL PBS and filter contents.

j. Aseptically remove the membrane filter and place onto TSA. Avoid trapping any air bubbles between the filter and agar surface.

k. Filter appropriate dilutions which yield countable numbers (up to 200 CFU per plate).

l. Carrier Sterility Control: add one sterile product carrier and one sterile stainless steel carrier to separate tubes containing 20 mL of TSB. Incubate at 36±1°C for 48±4 h and examine for growth. The acceptance criterion is lack of turbidity/growth in the tube.

m. Neutralizer Sterility Control: add 1 mL of neutralizer into 9 mL of TSB. Incubate at 36±1°C for 48±4 h and examine for growth. The acceptance criterion is lack of turbidity/growth in the tube.

n. Incubate plates from Unexposed Stainless Steel carriers at 36±1°C for 48±4 h. Incubate plates from Exposed Product, Exposed Stainless Steel, and Unexposed Product carriers at 36±1°C for 72±4 h.

   i. Monitor all plates (filters) after 24 h of incubation to facilitate appropriate timing for counting colonies.

   ii. For colony counts on filters in excess of 200 CFU, record as Too Numerous to Count (TNTC).

o. If isolated colonies are present, perform a Gram stain to assess one representative colony per carrier set (product exposed, product unexposed, stainless steel exposed, stainless steel unexposed).

p. If confluent growth is present, perform a streak isolation on the appropriate agar on growth taken from at least 1 representative filter per carrier set.

q. The results of the streak isolation plates should be consistent with characteristics in Table 5.

r. If additional verification of the test organism is required, perform further confirmatory analyses (e.g., VITEK or biochemical analyses) and isolation streaks on selective media.

**12. Virus Recovery**

a. Following the contact time, sequentially and aseptically transfer each carrier to a 50 mL conical tube containing 20 mL of the appropriate neutralizer solution. Remove and neutralize all carriers within 10 minutes

   i. The tube with the neutralizer and the carrier represents the $10^0$ dilution.

b. After all the carriers have been transferred into the neutralizer, vortex-mix for 30 s to suspend any surviving organism in the neutralizer. If necessary, to increase viral recovery, sonicate for 5 min±30 s at 45 Hz; refer to section 2cc for sonicator verification.

c. Initiate dilutions within 30 min after neutralization and vortex-mixing.

d. Initiate inoculation of cell line within 30 min of preparing the dilutions.

e. Titrate the samples for virus infectivity.

f. Plate a minimum of 80% of the volume (8 mL for 10 mL volumes, 16 mL for 20 mL volumes) of the $10^0$ vial and of each dilution tube.

g. Remove the growth medium from each well of the plate with a confluent monolayer of cells and replace with the maximum volume of the dilution tube (i.e., add 1 mL per well for a 24 well plate) working from most dilute to least dilute.

h. The elution steps for stainless steel control carriers are the same as for the product carriers; use 10-fold dilutions to achieve 4.0-5.0 logs virus particles/carrier.

i. For each test, use at least one well as a negative control (CGM alone) and one well as a positive growth control (e.g., one of the dilutions from a stainless steel control carrier).

j. If cytotoxicity was observed in the neutralization testing and/or on the cytotoxicity control, CGM may be removed from all wells in the affected dilutions at the appropriate time (one hour minimum). Wash the wells with pre-warmed PBS, then replace the PBS with fresh CGM.

k. Incubate test and control plates as appropriate for the test system.

l. Record all observations (presence/absence of viable virus particles) and use in calculations to estimate the log reduction based on the $TCID_{50}$ or MPN (most probable number) technique.

**13. Calculations/Data Analysis**

a. Use values with at least three significant figures when performing calculations (e.g., log density, mean log density). Report the final log reduction and difference in log reduction values with two significant figures.

b. For bacteria, calculate the Colony Forming Units (CFU)/carrier using the following equation:

$$\mathrm{Log}_{10}\left\{\left[\frac{\sum_{i=1}^{n}(Y_i)}{\sum_{i=1}^{n}(C_i \times D_i)}\right] \times V\right\}$$

where:

Y = CFU per filter,

C = volume filtered,

V = total volume of neutralizer,

D = $10^{-k}$,

k = dilution,

n = number of dilutions, and

i = lower limit of summation (the fewest number of dilutions).

c. For viruses, calculate the $TCID_{50}$/carrier or MPN/carrier. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier).

d. Calculate the mean log density (LD) of viable cells or virus particles for each microbe for the carrier sets in Lot 1 (Exposed Product (per chemical exposure/abrasion treatment, 3 total), Exposed Stainless Steel (per chemical exposure/abrasion treatment, 3 total), Unexposed Product, Unexposed Stainless Steel) as follows:

$Mean\ LD = \sum \frac{\mathrm{Log}_{10}(Carrier\ 1 + Carrier\ 2 + \ldots + Carrier\ X)}{X}$, where "X" refers to the total number of carriers assayed.

e. Calculate the mean LD of viable cells or virus particles for each microbe for the carrier sets in Lot 2 (Unexposed Product, Unexposed Stainless Steel) using the above equation.

f. For bacteria, when TNTC (Too Numerous to Count) values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and account for the dilution factor in the calculation.

g. Conduct additional calculations for Lot #1 and Lot #2; include in study report. See Tables 3 and 4 for calculations and outcome requirements.

**Table 3.** Additional calculations for Lot #1

| **LOG DIFFERENCE BETWEEN UNEXPOSED AND EXPOSED STAINLESS STEEL** | | **Outcome (Difference)** |
|---|---|---|
| Difference between Unexposed Stainless Steel and Exposed Stainless Steel | Mean LD Unexposed Stainless Steel – Mean LD Exposed Stainless Steel: Solution A | ≤ 0.5 |
| | Mean LD Unexposed Stainless Steel – Mean LD Exposed Stainless Steel: Solution B | ≤ 0.5 |
| | Mean LD Unexposed Stainless Steel – Mean LD Exposed Stainless Steel: Solution C | ≤ 0.5 |
| **LOG REDUCTION CALCULATIONS** | | **Outcome (LR)** |
| LR Unexposed Product | Mean LD Unexposed Stainless Steel – Mean LD Unexposed Product | ≥ 3.0 |
| LR Exposed Product | Mean LD Unexposed Stainless Steel – Mean LD Exposed Product: Solution A | ≥ 3.0 |
| | Mean LD Unexposed Stainless Steel – Mean LD Exposed Product: Solution B | ≥ 3.0 |
| | Mean LD Unexposed Stainless Steel – Mean LD Exposed Product: Solution C | ≥ 3.0 |
| **LOG DIFFERENCE BETWEEN UNEXPOSED AND EXPOSED PRODUCT** | | **Outcome (Difference)** |
| Difference between Unexposed Product and Exposed Product | Mean LR Unexposed Product – Mean LR Exposed Product: Solution A | ≤ 1.0 |
| | Mean LR Unexposed Product – Mean LR Exposed Product: Solution B | ≤ 1.0 |
| | Mean LR Unexposed Product – Mean LR Exposed Product: Solution C | ≤ 1.0 |

**Table 4.** Additional calculations for Lot #2

| **LOG REDUCTION CALCULATIONS** | | **Outcome (LR)** |
|---|---|---|
| LR Unexposed Product | Mean LD Unexposed Stainless Steel – Mean LD Unexposed Product | ≥ 3.0 |

## 14. References

a. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164.

b. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015.

c. ASTM Method E1482-12. Standard Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization (Reapproved 2017)

## Appendix A

### Preparation of Bacterial Frozen Stock Cultures

1. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from ATCC (or other reputable vendor) at least every 18 months.
    a. New frozen stock culture may be initiated one time using an existing, unexpired frozen stock culture as the source. Begin process at step 3 below, by streaking a loopful of the frozen stock culture onto 2 TSA plates.
2. Open ampule of freeze-dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly. Incubate broth culture at 36±1°C for 24±2 h.
3. At the end of the incubation timeframe, streak a loopful of the broth culture onto 2 TSA plates to obtain isolated colonies. Perform a streak isolation of the broth culture onto BAP as a purity check and streak the broth culture onto the appropriate selective media. Refer to appropriate selective media in Table 5. Incubate all plates for 24±2 h at 36±1°C.
    a. Record results at the end of the incubation timeframe. Refer to Table 5 for results on selective media and diagnostic characteristics of the test microbes.
4. From the TSA plates, select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of TSB. For *S. aureus*, select only golden yellow colonies. For *P. aeruginosa*, select colonies from each of the two possible phenotypes present. Spread plate 0.1 mL of the suspension onto each of 6-10 TSA plates. Incubate the plates for 24±2 h at 36±1°C. If necessary, to obtain more frozen stock cultures, a larger suspension (e.g., 2 mL) may be prepared using the same ratio of TSB (1 mL) to number of colonies (3-5 colonies).
    a. Using the TSB suspension, perform a streak isolation of the suspension onto a BAP as a purity check, and streak on the appropriate selective media (refer to Table 5).
    b. Incubate all plates for 24±2 h at 36±1°C. Record results. Refer to Table 5 for results on selective media and diagnostic characteristics of the test microbes.
5. After the incubation period, harvest growth from TSA plates by adding approximately 5 mL sterile cryoprotectant solution (TSB with 15% (v/v) glycerol) on the surface of each plate. Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL.
6. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.
7. Immediately after mixing, dispense 0.5-1.0 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.

a. For QC purposes, perform a streak isolation of the pooled culture onto a BAP as a purity check and streak on appropriate selective media (refer to Table 5).

b. Incubate all plates for 24±2 h at 36±1°C.

c. Record results.

d. After incubation, perform a Gram stain on growth from the BAP; observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

e. Conduct Vitek confirmation from growth taken from the BAP. Conduct VITEK according to the manufacturer's instructions.

f. Record all confirmation results.

8. Store the cryovials at approximately -80°C for a maximum of 18 months. These cultures are single-use only.

9. If the characteristics of the organism are not consistent with the information in Table 5 at any step in the process, or the Vitek profile is inconsistent with the organism, discard the cultures and re-initiate the process.

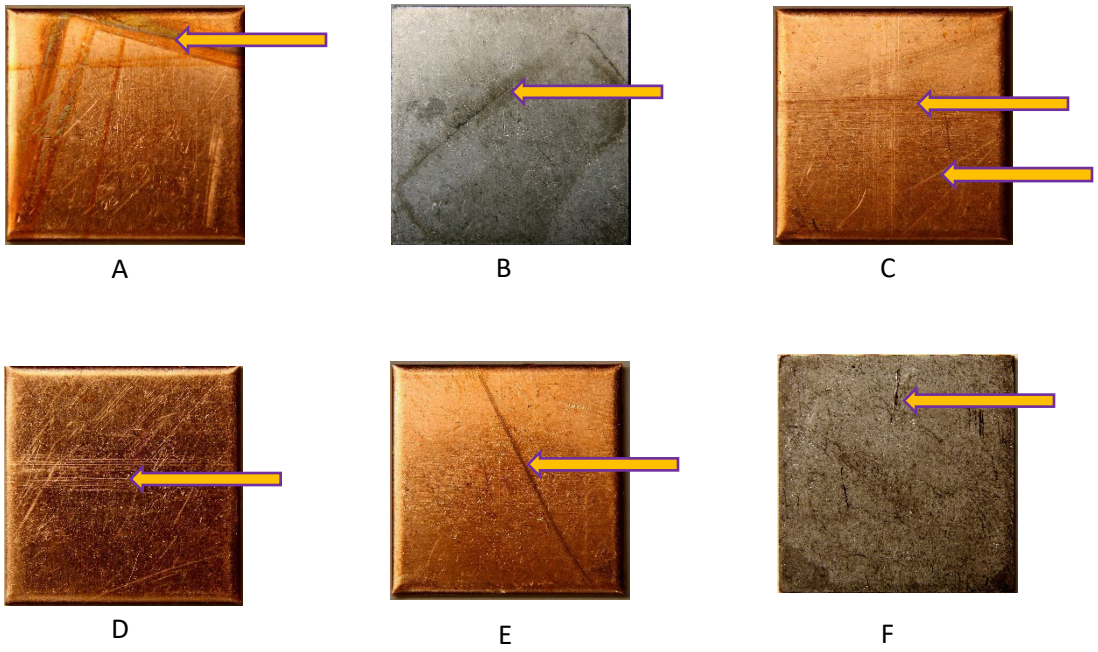
**Table 5.** Selective media and diagnostic characteristics for *P. aeruginosa* and *S. aureus*.

| Aspect | ***P. aeruginosa**** | ***S. aureus*** |
|---|---|---|
| Gram stain reaction | Negative | Positive |
| Mannitol Salt Agar Selective medium | N/A | Circular, small, yellow colonies, agar turning fluorescent yellow |
| Cetrimide Agar Selective medium | Circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green | N/A |
| Blood agar (BAP) | Flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic | Small, circular, yellow or white, glistening, beta hemolytic |
| | **Typical Microscopic Characteristics** | |
| Cell appearance | Straight or slightly curved rods, single polar flagella, rods formed in chains; 0.5-1.0 µm in diameter × 1.5-5.0 µm in length | Spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters; 0.5-1.0 µm in diameter |

*After 24±2 h (1) *P. aeruginosa* may display two phenotypes.

## Appendix B

## Examples of Failed Physically Screened Carriers



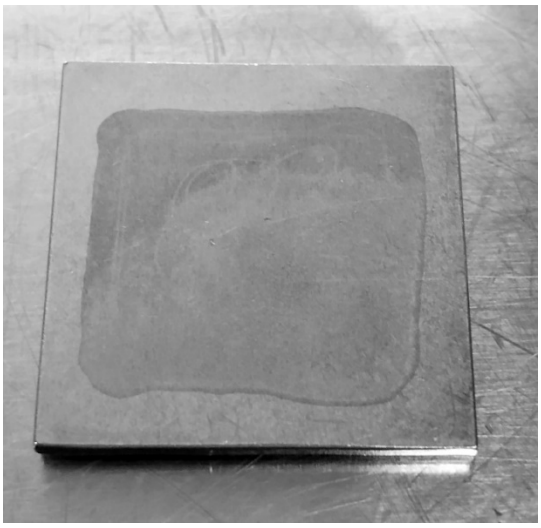

A and B fail due to discoloration of surface.

C and D fail due to surface scratches.

E and F fail due to deep gouge in surface.

## Appendix C

## Example of a Dry Inoculated Stainless Steel Carrier



## Appendix D

## Bacterial Neutralization Assay

1. The neutralization of the product carriers is confirmed in triplicate by using product carriers in neutralizer, neutralizer only (without carriers), and PBS only (used to compare counts from the neutralizer and product carriers).

2. **Preparation of the Test Organism**

    a. Refer to the preparation of test cultures section (8a-h) of for preparation of the test cultures. Conduct preliminary tests as necessary to determine appropriate dilution(s) of *Test Suspension A* (used to prepare *Test Suspension B*) to achieve the target challenge of 20-200 CFU per 20 µL.

    b. **Prepare Test Suspension A (without soil load)**. Serially dilute the microbial test suspension with PBS (e.g., through $10^{-4}$ or $10^{-5}$). Select appropriate dilutions of *Test Suspension A* so that after the addition of the soil load, *Test Suspension B* will achieve an average challenge of 20-200 CFU per 20 µL. Use *Test Suspension A* within 30 min of preparation.

        i. Two separate serial dilutions of *Test Suspension A* may be used to prepare two different concentrations of *Test Suspension B* to ensure at least one dilution with an average challenge of 20-200 CFU per 20 µL.

        ii. A calibration curve (OD @ 650nm) may be used to estimate the number of viable organisms in *Test Suspension A*.

    c. **Prepare Test Suspension B (with soil load)**. Prepare the 3-part soil load: using a vortex, mix each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, and 100 µL of mucin; then vortex-mix the solution. Add 340 µL of diluted *Test Suspension A* to the 160 µL of the soil load (SL) and vortex-mix for 10 s.

        i. Ensure at least one preparation of *Test Suspension B* provides an average challenge of 20-200 CFU per 20 µL.

3. **Neutralization Assay Components**

    a. ***Treatment 1: Neutralizer Effectiveness.*** Add a product carrier (one per market relevant lot) to each of three 50 mL conical tubes. At timed intervals, add 20 mL of neutralizer to each 50 mL conical tube and vortex-mix for 30 s on highest vortex setting. Immediately add 20 µL *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix D, section 4.

    b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 20 mL of neutralizer to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix D, section 4.

    c. ***Treatment 3: Titer Control.*** Add 20 mL of PBS to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix D, section 4.

4. **Processing and Recovery**

    a. Hold the mixtures from Appendix D, section 3 for 10±1 min at room temperature (21±3°C).

    b. At the conclusion of the holding period, vortex-mix each tube.

    c. Initiate filtration as soon as possible (e.g., within 30 min).

    d. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS. Filter each mixture through a separate, pre-wetted 0.2 µm PES membrane filter. Apply vacuum to filter contents; leave the vacuum on for the duration of the filtration process regardless of filtration apparatus used (e.g., filter manifold, single filter unit).

    e. Wash each tube with ~20 mL PBS and vortex-mix; filter the wash through the same filter membrane. Finish the filtering process by rinsing the inside of the funnel unit with ~20 mL PBS and filter the rinsing liquid through the same filter membrane.

    f. Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the TSA. Avoid trapping air bubbles between the filter and the agar surface.

    g. Incubate plates at 36±1°C for 48±4 h and count the colonies.

    h. Incubate plates with no growth or few colonies an additional 24±4 h and count the number of colonies.

5. **Data Analysis/Calculations**

    a. Compare the average CFU of the **Titer Control** with the average CFU of the **Neutralizer Toxicity Control** and **Neutralizer Effectiveness** treatment. Determine the percent difference in CFU.

    b. For determining the suitability of the neutralizer, ensure that the average CFU in the **Neutralizer Toxicity Control** is at least 50% of the **Titer Control**. A count lower than 50% indicates that the neutralizer is harmful to the test organism.

        i. Average CFU for the **Neutralizer Toxicity Control** that are higher than the **Titer Control** (e.g., 120% of the **Titer Control**) are also deemed valid.

    c. To verify effectiveness of the neutralizer, the average number of CFU in the **Neutralizer Effectiveness** treatment is at least 50% of the **Titer Control**.

        i. Average CFU for the **Neutralizer Effectiveness** treatment that are higher than the **Titer Control** (e.g., 120% of the **Titer Control**) are also deemed valid.

    d. If the criteria are not met, verify another neutralizer or mixture of neutralizers.

## Appendix E

## Cytotoxicity Determination

Prior to performing the neutralization assay, ensure the proposed neutralizer, neutralizer and test chemical, and the soil used do not impact the quality of the cell line by performing the following:

1. **Neutralizer Effect on Cell Line (for neutralizers other than CGM with 2% FBS).**
   a. Add 0.5 mL of the proposed neutralizer to 4.5 mL CGM with 2% (v/v) FBS, equilibrated to 37±1°C (this is the $10^{-1}$ dilution). It is suggested to do further dilutions out to $10^{-2}$ or $10^{-3}$ depending on the expected cytotoxicity of the neutralizer.
   b. Remove the CGM from the wells of a 24 well plate with an 80-95% confluent monolayer of cells and add 1 mL per well of the neutralizer plus CGM solution. Plate at least 4 wells per dilution. Have at least one well as a negative control (e.g., CGM with 2% FBS alone).
   c. Incubate plate as appropriate and observe closely for cytotoxicity.
   d. If cytotoxicity is observed after one hour, remove the media in a single well of the affected dilution, rinse once with pre-warmed DPBS (the DPBS wash step may be omitted if the cytotoxicity is mild), and replace media.
   e. If cell death occurs in under one hour, the neutralizer cannot be tested.
   f. The effect of the media change in the single well can be compared to the other wells in the dilution and the negative control. If cytotoxicity cannot be overcome with washing and replacing of media, column filtration (e.g., Sephadex) may be used in future testing. See section 14c for further information on column filtration.
2. **Neutralizer Plus Test Carrier Effect on Cell Line.**
   a. Add one test carrier to 20 mL of neutralizer, equilibrated to 21±3°C, and vortex 2-3 seconds. Let this solution sit at room temperature for 10 minutes.
   b. Add 1.0 mL of this solution to 9 mL CGM with 2% (v/v) FBS, equilibrated to 37±1°C (this is the $10^{-1}$ dilution). It is suggested to do further dilutions out to $10^{-2}$ depending on the expected cytotoxicity.
   c. Remove the CGM from the wells of a 24 well plate with a confluent monolayer of cells and add 1 mL per well of the neutralizer plus test carrier and dilutions. Plate at least 8 wells for the $10^{0}$ dilution, 6 wells for the $10^{-1}$ dilution, and 4 wells for the $10^{-2}$ dilution. Extra wells will be needed to observe the effect of no media changes or for further media changes as needed.
   d. For highly toxic test chemicals, washing the cells with pre-warmed DPBS before the addition of CGM with 2% FBS will help remove cytotoxicity.
   e. Have at least one well on each plate as a negative control (e.g., CGM with 2% (v/v) FBS alone).
   f. At a minimum, change the media in the wells as outlined below. Change the media at the lower time interval if they look more toxic. Other media changes can be made at other times if necessary.

i. For the $10^0$ dilution:

i. On the day of the test, change two wells 1-2 hours (1-hour minimum) after the neutralized test chemical mixture was added to the cells.

ii. Change two more additional wells 3-5 hours after the neutralized test chemical mixture was added to the cells.

iii. The following day, change one 1-2 hour well, one 3-5 hour well, and one previously unchanged well, plus one of the two previously changed well.

ii. For the $10^{-1}$ dilution:

i. On the day of the test, change tow wells 3-5 hours after the neutralized test chemical mixture was added to the cells.

ii. The following day, change one 3-5 hour well and one previous unchanged well.

iii. For the $10^{-2}$ dilution:

i. On the day after the test, change one well.

g. Incubate the plate as appropriate and observe the cells for cytotoxicity. The test cells should be compared to the negative control cells to determine toxicity.

h. Score the cells as toxic or non-toxic in each in each test conditions.

i. Identify the test condition that removed the cytotoxicity and use that condition for further neutralization and efficacy testing. Use the test condition that allows the media to stay on the cells for as long as possible.

i. **Example:** In the $10^0$ dilution, if the unchanged wells are toxic, but both the 1 hour and 4 hour media changes are non-toxic, change the media in the $10^0$ dilutions after 4 hours in all future testing.

j. If cell death occurs in under one hour, that test condition cannot be used.

k. Cytotoxicity past the $10^{-1}$ dilution is unacceptable for testing. Alternative neutralizers or column filtration (e.g., Sephadex) may be used to mitigate cytotoxicity. See section 14c for further information on column filtration.

3. **3-Part Soil Effect on Cell Line.**

a. Make the 3-part soil (see section 9e but withhold the virus).

b. Add 10 µL of the soil to 20 mL of CGM, equilibrated to 37±1°C.

c. Remove the CGM from the cells and add 1 mL of this solution to 4 wells on a 24 well plate with a confluent monolayer of cells. Have at least one well as a negative control (e.g., CGM alone).

d. Incubate plate as appropriate and observe daily for cytotoxicity. No cytotoxicity should be observed.

## Appendix F
## Viral Neutralization Assay

1. Perform the neutralization assay prior to testing to demonstrate the neutralizer's ability to inactivate the product carrier.
2. Select a neutralizing medium that is not inhibitory to the virus and is not cytotoxic to the cells. The acceptance criteria for acceptable neutralization are 0.5 log differences between the neutralization effectiveness, neutralization toxicity control, and titer control. Interaction between the neutralizer and product and its effect on the cell line must be determined prior to testing.
3. **Prepare *Test Suspension A***. Dilute the virus stock suspension in CGM to achieve an average recovered concentration of approximately 2-3 logs (i.e., 100-1000 virus particles) per vessel for the Titer Control sample. To achieve this, dilute the virus stock suspension through $10^{-4}$ (or as necessary).
4. **Prepare *Test Suspension B***. Prepare the soil load: vortex each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, 100 µL of mucin, and add 340 µL of *Test Suspension A* (0.5 mL total volume) and mix well. Use *Test Suspension B* within 30 minutes of preparation.
5. **Neutralization Treatments**
    a. ***Treatment 1: Neutralizer Effectiveness.*** Add a product carrier (one per market relevant lot) to each of three 50 mL conical tubes. At timed intervals, add 20 mL of neutralizer to each 50 mL conical tube and vortex-mix for 30 s on highest vortex setting. Immediately add 20 µL *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix F, section 6.
    b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 20 mL of neutralizer to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix F, section 6.
    c. ***Treatment 3: Titer Control.*** Add 20 mL CGM to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix F, section 6.
        i. Note: Steps should be conducted at timed intervals (e.g., 30 s) to ensure consistent time of contact.
6. **Processing and Recovery**
    a. Hold the mixtures from Appendix F, section 5 for 10±1 min at room temperature (21±3°C).
    b. At the conclusion of the holding period, vortex each tube for 3-5 s. Serially dilute the sample as needed (e.g., remove 1 mL of sample and dilute in 9 mL of CGM).

i. Initiate dilution and plating as soon as possible (e.g., within 5 minutes). Two analysts are recommended to perform vortexing and dilution steps to reduce holding time after vortexing.

ii. Titrate the samples for virus infectivity using the appropriate cell line – plate a minimum of 80% of the $10^0$ vessel and all dilutions.

iii. For each well plated, add the maximum volume of the well (i.e., add 1 mL per well for a 24 well plate).

iv. Note: If any $10^0$ (vessel) dilution is used that does not contain CGM (e.g., Treatment 2 with proposed neutralizer), allow it to adsorb on the cells for 1 hr, then remove and replace with fresh CGM.

7. If cytotoxicity was observed in pre-neutralization testing, CGM may be removed from all wells in the affected dilutions at the appropriate time (one hour minimum), the wells washed with pre-warmed PBS, and then the PBS replaced with fresh CGM. Follow the same procedure for dilutions from the control carriers.
8. Incubate test and control plates as appropriate for the test system.
9. For the neutralizer to be considered effective:
    a. Ensure that the recovered virus in the **Titer Control** using *Test Suspension B* is between approximately 2-3 logs per vessel.
    b. The recovered virus in the **Neutralizer Effectiveness** treatment is within 0.5 logs of the **Titer Control**; this verifies effective neutralization. A log reduction greater than 0.5 logs indicates that the neutralizer was not effective. Note: a value higher than the **Titer Control** is also deemed valid.
    c. The recovered virus in the **Neutralizer Toxicity Control** is within 0.5 logs of the **Titer Control**. A log reduction greater than 0.5 logs indicates that the neutralizer is harmful to the test system. Note: a value higher than the **Titer Control** is also deemed valid.
10. All criteria in Appendix F, section 9 must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified.

## Appendix G

## Recipe for Synthetic broth

**Solution A:** Dissolve the following in 500 mL $H_2O$ containing 18 mL 1 N NaOH:

- 0.05 g L-cystine
- 0.37 g DL-methionine
- 0.4 g L-arginine×HCl
- 0.3 g DL-histidine
- 0.85 g L-lysine×HCl
- 0.21 g L-tyrosine
- 0.5 g DL-threonine
- 1.0 g DL-valine
- 0.8 g L-leucine
- 0.44 g DL-isoleucine
- 0.06 g glycine
- 0.61 g DL-serine
- 0.43 g DL-alanine
- 1.3 g L-glutamic acid×HCl
- 0.45 g L-aspartic acid
- 0.26 g DL-phenylalanine
- 0.05 g DL-tryptophan
- 0.05 g L-proline

**Solution B:** Dissolve the following in 500 mL $H_2O$:

- 3.0 g NaCl
- 0.2 g KCl
- 0.1 g $MgSO_4 \times 7\ H_2O$
- 1.5 g $KH_2PO_4$
- 4.0 g $Na_2HPO_4$
- 0.01 g thiamine×HCl
- 0.01 niacinamide

Mix Solutions A and B, and steam sterilize 20 min at 121°C.
Final pH should be 7.1±0.1.